

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 15/10, C07K 16/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/43507</b> <b>(43) International Publication Date:</b> 27 July 2000 (27.07.00)
<b>(21) International Application Number:</b> PCT/EP00/00296 <b>(22) International Filing Date:</b> 13 January 2000 (13.01.00) <b>(30) Priority Data:</b> 99300351.6 19 January 1999 (19.01.99) EP <b>(71) Applicant (for AE AU BB CA CY GB GD GH GM IE IL KE LC LK LS MN MW NZ SD SG SL SZ TT TZ UG ZA ZW only):</b> UNILEVER PLC (GB/GB); Unilever House, Blackfriars, London EC4P 4BQ (GB). <b>(71) Applicant (for all designated States except AE AU BB CA CY GB GD GH GM IE IL IN KE LC LK LS MN MW NZ SD SG SL SZ TT TZ UG ZA ZW):</b> UNILEVER NV [NL/NL]; Weena 455, NL-3013 AL Rotterdam (NL). <b>(71) Applicant (for IN only):</b> HINDUSTAN LEVER LIMITED [IN/IN]; Hindustan Lever House, 165/166 Backbay Reclamation, Maharashtra, 400 020 Mumbai (IN).		<b>(72) Inventors:</b> FRENKEN, Leon, Gerardus, Joseph; Unilever Research Colworth, Colworth House, Sharnbrook, Bedford, Bedfordshire MK44 1LQ (GB). VAN DER LOGT, Cornelis, Paul, Eric; Unilever Research Colworth, Colworth House, Sharnbrook, Bedford, Bedfordshire MK44 1LQ (GB). <b>(74) Agents:</b> EVANS, Jacqueline, Gail, Victoria et al.; Unilever PLC, Patent Department, Colworth House, Sharnbrook, Bedford, Bedfordshire MK44 1LQ (GB). <b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> METHOD FOR PRODUCING ANTIBODY FRAGMENTS  <b>(57) Abstract</b>  An expression library comprising a repertoire of nucleic acid sequences cloned from a non-immunised source, each nucleic acid sequence encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains (VHH) wherein the extent of sequence variability in said library is enhanced compared to the corresponding naïve expression library by introducing mutations in one or more of the complementarity determining regions (CDRs) of said nucleic acid sequences or by generating alternative combinations of CDR and framework sequences not naturally present in the naïve library repertoire. Also disclosed is the use of such a library in preparing antibodies, more particularly antibody fragments.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LJ	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

METHOD FOR PRODUCING ANTIBODY FRAGMENTSFIELD OF THE INVENTION

5 The present invention relates to an expression library comprising a repertoire of nucleic acid sequences derived from the natural sequence repertoire but modified to enhance the extent of sequence variability, each said nucleic acid sequence encoding at least part of a variable domain of a heavy chain  
10 derived from an immunoglobulin naturally devoid of light chains and its use in producing antibodies, or more particularly fragments thereof.

In particular, the invention relates to a method for the  
15 preparation of antibodies or fragments thereof, having binding specificity for a target antigen which avoids the need for the donor previously to have been immunised with the target antigen.

BACKGROUND TO THE INVENTION

20 Monoclonal antibodies, or binding fragments thereof, have traditionally been prepared using hybridoma technology (Kohler and Milstein, 1975, Nature 256, 495). More recently, the application of recombinant DNA methods to generating and  
25 expressing antibodies has found favour. In particular, interest has concentrated on combinatorial library techniques with the aim of utilising more efficiently the antibody repertoire.

The natural immune response *in vivo* generates antigen-specific  
30 antibodies via an antigen-driven recombination and selection process wherein the initial gene recombination mechanism generates low specificity, low-affinity antibodies. These clones can be mutated further by antigen-driven hypermutation of

the variable region genes to provide high specificity, high affinity antibodies.

Approaches to mimicking the first stage randomisation process which have been described in the literature include those based on the construction of 'naive' combinatorial antibody libraries prepared by isolating panels of immunoglobulin heavy chain variable (VH) domains and recombining these with panels of light variable chains (VL) domains (see, for example, Gram et al, Proc. Natl. Acad. Sci. USA, 89, 3576-3580, 1992). Naive libraries of antibody fragments have been constructed, for example, by cloning the rearranged V-genes from the IgM RNA of B cells of un-immunised donors isolated from peripheral blood lymphocytes, bone marrow or spleen cells (see, for example, Griffiths et al, EMBO Journal, 12(2), 725-734, 1993, Marks et al, J. Mol. Biol., 222, 581-597, 1991). Such libraries can be screened for antibodies against a range of different antigens.

In combinatorial libraries derived from a large number of VH genes and VL genes, the number of possible combinations is such that the likelihood that some of these newly formed combinations will exhibit antigen-specific binding activity is reasonably high provided that the final library size is sufficiently large. Given that the original B-cell pairings between antibody heavy and light chain, selected by the immune system according to their affinity of binding, are likely to be lost in the randomly, recombined repertoires, low affinity pairings would generally be expected. In line with expectations, low affinity antibody fragments (Fabs) with  $K_d$ s of  $10^4$ - $10^5$  M<sup>-1</sup> for a progesterone-bovine serum albumin (BSA) conjugate have been isolated from a small ( $5 \times 10^6$ ) library constructed from the bone marrow of non-immunised adult mice (Gram et al, see above).

Antibody fragments of higher affinity ( $K_d$ s of  $10^6$ - $10^7$  M<sup>-1</sup> range) were selected from a repertoire of  $3 \times 10^7$  clones, made from the

- peripheral blood lymphocytes of two healthy human volunteers (Marks et al, see above) comprising heavy chain repertoires of the IgM (naive) class. These were combined with both Lamda and Kappa light chain sequences, isolated from the same source.
- 5 Antibodies to more than 25 antigens were isolated from this library, including self-antigens (Griffiths et al, see above) and cell-surface molecules (Marks et al, Bio/Technology, 11, 1145-1149, 1993).
- 10 The second stage of the natural immune response, involving affinity maturation of the selected specificities by mutation and selection has been mimicked *in-vitro* using the technique of random point mutation in the V-genes and selecting mutants for improved affinity.
- 15 Recently, the construction of a repertoire of  $1.4 \times 10^{10}$  scFv clones, achieved by 'brute force' cloning of rearranged V genes of all classes from 43 non-immunised human donors has been reported (Vaughan et al 1996) and Griffiths et al, see above.
- 20 Antibodies to seven different targets (including toxic and immunosuppressant molecules) were isolated, with measured affinities all below 10nM.

The main limitation in the construction of combinatorial  
25 libraries is their size, which consequently limits their complexity. Evidence from the literature suggests that there is a direct link between library size and diversity and antibody specificity and affinity (see Vaughan et al, Nature Biotechnology, 14, 309-314, 1996), such that the larger (and  
30 more diverse) the library, the higher the affinity of the selected antibodies.

The optimisation of binding affinity through random recombination of a heavy and light chain in combinatorial  
35 libraries is complicated by sequence variations in the two

framework regions, (i.e. the parts of the variable domains that serve as a scaffold in supporting the regions of hypervariability which are in turn termed the complementary determining regions or CDRs.

5

Only some combinations of framework sequences are compatible with the folding and interaction required for the correct orientation of the 6 CDRs that is necessary for good binding affinity. Consequently, conventional combinatorial libraries are likely to contain a high percentage of molecules that are non-functional.

The affinity of antibodies may also be improved by the process of "chain shuffling", whereby a single heavy or light chain is recombined with a library of partner chains (Marks et al, Bio/Technology, 10, 779-782, 1992).

EP-A-0368684 (Medical Research Council) discloses the construction of expression libraries comprising a repertoire of nucleic acid sequences each encoding at least part of an immunoglobulin variable domain and the screening of the encoded domains for binding activities. It is stated that repertoires of genes encoding immunoglobulin variable domains are preferably prepared from lymphocytes of animals immunised with an antigen. The isolation of single VH domains having antigen binding activities, facilitated by immunisation, is exemplified (see Example 6).

These results illustrate that although the VH part alone of a classical antibody binding domain can exhibit binding activity, the specificity and affinity are generally very low. This may be explained by the absence of the functional involvement of the missing light chain such that only half of the intended binding pocket is present, leading to binding with related or homologous targets.

EP-A-0368684 further describes the cloning of heavy chain variable domains with binding activities generated by mutagenesis of one or each of the CDRs. The preparation of a repertoire of CDR3s is described by using "universal" primers based in the flanking sequences, and likewise repertoires of other CDRs singly or in combination. These synthetic mutant VH clones can then be recombined with VL chains to produce a synthetic combinatorial library.

10

Construction of libraries by such synthetic recombinatorial techniques produces a repertoire of molecules that collectively exhibit a good degree of binding diversity, wherein the variability is focussed into the sequences that encode the CDRs of each chain. However this technique does not overcome the problems previously discussed with respect to random recombination of heavy and light chains and production of non-functional molecules. Furthermore there are still six separate regions (3 CDRs in VH and another 3 in VL) determining the binding capacity of the molecule hence the repertoire of possible binding variants is encoded within a rather diffuse stretch of coding sequence, thus making a focussed approach to altering the binding affinity of these binding domains very difficult.

20

There remains a continuing need for the development of improved methods for constructing libraries of immunoglobulin binding domains. In particular, it would be desirable to avoid the recombination of heavy and light chains thereby preventing the formation of molecules that are non-functional following recombination.

30

It would also be an advantage to reduce the number of hypervariable residues in the binding domain as this would allow

a more complete repertoire of possible binding variants to be obtained.

WO 94/4678, Casterman et al, describes immunoglobulins capable  
5 of exhibiting the functional properties of conventional (four-  
chain) immunoglobulins but which comprise two heavy polypeptide  
chains and which furthermore are devoid of light polypeptide  
chains. Fragments of such immunoglobulins, including fragments  
corresponding to isolated heavy chain variable domains or to  
10 heavy chain variable domain dimers linked by the hinge  
disulphide are also described. Methods for the preparation of  
such antibodies or fragments thereof on a large scale comprising  
transforming a mould or yeast with an expressible DNA sequence  
encoding the antibody or fragment are described in patent  
15 application WO 94/25591 (Unilever).

The immunoglobulins described in WO 94/4678, which may be  
isolated from the serum of Camelids, do not rely upon the  
association of heavy and light chain variable domains for the  
20 formation of the antigen-binding site but instead the heavy  
polypeptide chains alone naturally form the complete antigen  
binding site. These immunoglobulins, hereinafter referred to as  
"heavy-chain immunoglobulins" are thus quite distinct from the  
heavy chains derived from conventional (four-chain)  
25 immunoglobulins. Heavy chains from conventional immunoglobulins  
contribute part only of the antigen-binding site and require a  
light chain partner, forming a complete antigen binding site,  
for optimal antigen binding.

30 As described in WO 94/4678, heavy chain immunoglobulin  $V_H$   
regions isolated from Camelids (hereinafter VHH domains) which  
form a complete antigen binding site and thus constitute a  
single domain binding site differ from the  $V_H$  regions derived  
from conventional four-chain immunoglobulins in a number of  
35 respects, notably in that they have no requirement for special



features for facilitating interaction with corresponding light chain domains. Thus, whereas in conventional (four-chain) immunoglobulins the amino acid residue involved in the  $V_H/V_L$  interaction is highly conserved and generally apolar leucine, in Camelid derived  $V_H$  domains this is replaced by a charged amino acid, generally arginine. It is thought that the presence of charged amino acids at this position contributes to increasing the solubility of the camelid derived  $V_H$ . A further difference which has been noted is that one of the CDRs of the heavy chain immunoglobulins of WO 94/4678, the CDR<sub>3</sub>, may contain an additional cysteine residue associated with a further additional cysteine residue elsewhere in the variable domain. It has been suggested that the establishment of a disulphide bond between the CDR<sub>3</sub> and the remaining regions of the variable domain could be important in binding antigens and may compensate for the absence of light chains.

cDNA libraries composed of nucleotide sequences coding for a heavy-chain immunoglobulin and methods for their preparation are disclosed in WO 94/4678. It is stated that these immunoglobulins have undergone extensive maturation *in vivo* and the V region has naturally evolved to function in the absence of the light chain variable domain. It is further suggested that in order to allow for the selection of antibodies having specificity for a target antigen, the animal from which the cells used to prepare the library are obtained should be pre-immunised against the target antigen. No examples of the preparation of antibodies are given in the specification of WO 94/4678. The need for prior immunisation is also referred to in Arabi Ghahroudi et al (FEBS Letters, 414, (1997), 521-526).

Davies et al (Bio/Technology, 13, 475-479, 1995) describe an approach to the construction of a library of binding domains based on a modified human  $V_H$  domain which is intended to mimic a camelid  $V_{HH}$  domain. This method involves replacement of the

sequence segment encoding one of the CDRs by random, synthetic sequences. Although it was possible to isolate domains with selected antigen binding properties from the resulting library, these were generally characterised by poor binding affinity and  
5 specificity for protein antigens. The results would not seem, therefore, to recommend the further application of this type of approach.

The present invention relates to an expression library  
10 comprising a repertoire of synthetic or semi-synthetic nucleic acid sequences, not cloned from an immunised source, wherein said nucleic acid sequences are derived from immunoglobulins that are naturally devoid of light chains.

#### 15 SUMMARY OF THE INVENTION

This invention is based on the unexpected finding that high affinity, high specificity antibodies or fragments thereof capable of binding either to protein or small molecule antigens  
20 can be obtained from a non-immunised camelid source provided that random mutagenesis of one or more CDRs is carried out, or that alternative combinations of existing CDRs are generated, in order to increase the extent of sequence variability in the antibody repertoire.

25

The present invention therefore provides an expression library comprising a repertoire of nucleic acid sequences, which sequences are not cloned from an immunised source, each nucleic acid sequence encoding at least part of a variable domain of a  
30 heavy chain derived from an immunoglobulin naturally devoid of light chains wherein the extent of sequence variability in said library is enhanced compared to the corresponding naïve expression library by introducing mutations in one or more of the complementarity determining regions (CDRs) of said nucleic  
35 acid sequences or by random recombination of fragments of said

nucleic acid sequences, thereby generating alternative combinations of CDR and framework sequences not naturally present in the naïve library repertoire.

## 5 BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows a schematic representation of the assembly strategy expression for the library, wherein

- 10 Step 1 illustrates the isolation of the framework regions.  
Step 2A-C illustrate attaching the variable CDR regions to FR2, FR3 and FR4 respectively.  
Step 3 involves linking the FR1 to the CDR1-FR2 encoding fragments.  
15 Step 4 involves linking the FR1-CDR1-FR2 to the CDR2-FR3 fragments.  
Step 5A-D depicts the final 'full length' VHH fragment assemblies.
- 20 Figure 2 illustrates variability designed into the CDR regions of a synthetic library (where variability is designed into all three CDR) and semi-synthetic library (where only the CDR3 varies).

## 25 DEFINITION OF TERMS

The term "naive library" refers to a collection of nucleic acid sequences encoding the naturally occurring VHH repertoire from a non-immunised source (see, Example 1).

30

The term "synthetic library" refers to a collection of nucleic acid sequences herein referred to as synthetic nucleic acid sequences, encoding single heavy chain antibodies or fragments thereof in which all CDR regions have undergone some form of  
35 rearrangement.

The term "semi-synthetic library" refers to a collection of nucleic acid sequences encoding single heavy chain antibodies or fragments thereof in which at least one CDR region retains  
5 natural variability and at least one CDR region has undergone some form of controlled rearrangement. Preferably in the semi-synthetic library the CDR to be randomised or mutagenised is the CDR-3.

10 As used herein, the term "antibody" refers to an immunoglobulin which may be derived from natural sources or synthetically produced, in whole or in part. The terms "antibody" and "immunoglobulin" are used synonymously throughout the specification unless indicated otherwise.

15

An "antibody fragment" is a portion of a whole antibody which retains the ability to exhibit antigen binding activity.

The term "VHH" refers to the single heavy chain variable domain  
20 antibodies of the type that can be found in Camelid mammals which are naturally devoid of light chains; synthetic and naive VHH can be construed accordingly.

The term "CDR" refers to the complementary determining region of  
25 the antibody structure.

The term "library" refers to a collection of nucleic acid sequences.

30 The term "repertoire," again meaning a collection, is used to indicate genetic diversity.

The term "framework region" is used herein to refer to the nucleic acid sequence regions of an antibody molecule that  
35 encode the structural elements of the molecule.

The term "anchor regions" refers to nucleic acid sequences that show homology to part of the nucleic acid sequence of the framework region or class of framework regions, such that  
5 primers based on these anchor region sequences can be used to amplify the framework regions that are present in the naive library. In the present invention the primers based on the anchor regions were able to identify at least  $10^6$  different framework region sequences which could be divided into 5  
10 different classes of fragments.

#### DETAILED DESCRIPTION OF THE INVENTION

The invention is based on the unexpected finding that the  
15 development of an expression library consisting of VHH domains derived from an immunoglobulin naturally devoid of light chains in which one or more of the three CDRs have been modified to enhance the extent of their sequence variability provides an effective and superior source of high affinity and high  
20 specificity antibodies, or fragments thereof when compared to conventional dual chain antibody expression libraries.

The heavy chain variable domains (VHH) for use according to the invention may be derived from any immunoglobulin naturally  
25 devoid of light chains, such that the antigen-binding capacity and specificity is located exclusively in the heavy chain variable domain.

Preferably, the heavy chain variable domains may be obtained  
30 from camelids (as described in WO 94/4678, above), especially *Lamas* (for example *Lama Glama*, *Lama Vicugia* or *Lama Paccos*) or from *Camelus* (for example *Camelus dromedarius* or *Camelus bactrionus*). Suitable sources include lymphoid cells,

especially peripheral blood lymphocytes, bone marrow cells and spleen cells.

In one aspect of the invention, the framework regions of the VHH domains may conveniently be derived from a naïve library of VHH domains. This allows the natural variability in these sequence segments to be reflected in the expression library. To achieve this, the present invention has utilised information on 200 clones selected at random from a naïve library of VHH. This has allowed the identification of "anchor-regions" i.e. sequences which are highly conserved within the naïve clones and which are thus able to provide the basis for the design of primers capable of amplifying most if not all sequence variants of the framework regions, present in the naïve library. To the extent that the framework sequences have some variability in the naïve library it is desirable also to retain this in the modified library according to the invention.

This embodiment of the invention therefore provides for the use of a naïve VHH expression library derived from a non-immunised source for the construction of a synthetic or semi-synthetic VHH expression library.

Although in the present invention 200 clones have been randomly selected as a basis for framework primer design, it will be appreciated that it would be within the capacity of the person skilled in the art to design framework primers based on the homology of anchor regions with framework sequences taken from a much smaller number of clones, indeed it is not outside the capacity of the person skilled in the art to design framework primers from the sequence of a single naïve cDNA clone. However, it remains the case that optimum anchor regions can most accurately be pinpointed by analysis of the homology of a large number of clones.

35

Therefore taking sequence homology data obtained from the randomly selected individual clones cDNA framework primers were designed (see Table 1);

- 5    i.    to have sequences complementary to the anchor regions;
- ii. to have a melting temperature preferably of at least 50°C,  
         to facilitate annealing.
- 10   A large number of framework region clones could be generated in  
     this way comprising 5 different types of fragments i.e. FR-1/FR-  
     2A/FR-2B/FR-3/FR-4.

15   In another aspect of the invention, variability of the CDRs  
     derived from the naïve repertoire is enhanced by mutation of at  
     least some of the residues they comprise. Preferably this  
     process introduces regions of random sequence into at least some  
     of the CDRs. According to a particular embodiment, the CDR  
20   sequence in each individual clone is replaced by a synthetic  
     nucleic acid sequence. Conveniently, the mutagenesis of the  
     CDRs may be achieved by the method of overlap extension using  
     primers which contain at each end sequences that are  
     complementary or homologous to the anchor regions that form the  
25   basis of the framework region primers listed in Table 1 and, in  
     between, random or partly random sequences that will ultimately  
     encode the CDR regions. The nucleic acid sequences of the  
     synthetically modified CDR primers are listed in Table 2.

30   It is important when designing the CDR primers also to take into  
     account sequence homology within the CDR regions which was  
     observed in the sequence data from the naïve clones, as the  
     amino acids concerned are thought to play a structural role in  
     the VHH. It is desirable that highly conserved sequences within  
     the CDRs, that is, residues that are conserved amongst a  
35   substantial proportion of the VHH domains in the naïve

repertoire, should be retained in the synthetically modified primers, and excluded as targets for mutagenesis.

5 Splicing by overlap extension follows: This is a modification of the polymerase chain reaction which has been used to generate gene fusions at very specific positions. It is based on the ability to fuse and amplify two DNA fragments containing homologous sequences i.e. 'anchors' around the fusion point.

10 For the preparation of a 'synthetic' expression library, CDR primers incubated with framework region fragments will anneal at their complementary ends and fuse to generate randomised framework-CDR encoding fragments (see Figure 1, step 2A, B, C). This process yields CDR-1/FR-2, CDR-2/FR-3 and CDR-3/FR-4 fusion  
15 fragments. Two of these fragments are then fused (see Figure 1, step 3), and so forth (steps 4 and 5).

There then follows a denaturation step after which the fragments can be further annealed at the 'anchor-regions' and extended  
20 yielding the fused, double stranded gene product. If required this reaction can be followed by the PCR reaction amplifying the quantity of fused gene material. This method can easily be extended to fuse three or more fragments.

25 Splicing by overlap extension allows the linking of the fusion fragments at specific positions to produce a fully assembled HC-V gene which can be cloned into a suitable phage display vector such as the vector pHEN.5 using restriction enzymes such as SfiI/NotI.

30

It will be appreciated that other methods of introducing mutations, preferably including random or partially random sequences, into the CDRs would also be applicable. Such methods include, for example, cassette mutagenesis or the use of error-  
35 prone 'mutator' strains as bacterial hosts.



In one aspect, the present invention provides the use of a naive VHH expression library, not derived from an immunised source for the construction of a synthetic VHH expression library  
5 characterised in that all CDRs undergo a degree of sequence modification.

In another aspect, only one or two of the CDRs in the heavy chain variable domain are provided with enhanced sequence  
10 variability by the introduction of random synthetic sequences. Thus for the preparation of a 'semi-synthetic' library the FR-1/CDR-1/FR-2/CDR-2/FR-3 genes from the naive library were assembled with CD-3/FR-4 fusion fragments and cloned into the phage display vector PHEN.5 as SfiI/NotI fragments.

15 This aspect of the invention therefore comprises the use of a naive VHH expression library derived from a non-immunised source for the construction of a semi-synthetic VHH expression library characterised in that one or two CDRs undergo a degree of  
20 sequence modification.

In a further aspect of the invention, VHH domains with alternative combinations of three CDR sequences, which would not have been present in the unmodified naive library, may be  
25 generated by random recombination of fragments of VHH domain sequences derived from a naive library. Optionally, this recombination process may be combined with mutagenesis of one or more of the CDRs, along the lines discussed above.

30 A further embodiment of the present invention resides in a method of preparing an expression library as disclosed above, comprising the steps of:

- (i) taking sequence data obtained from a number of cDNA clones  
35 randomly selected from a naive VHH library;

(ii) identifying a series of 'anchor regions' which show substantially conserved homology within said sequence data and on which basis framework primers with a capacity to amplify framework regions of the naive library target DNA can be constructed;

(iii) amplification from a non-immunised source of a maximal number of different framework regions using primers from step (ii);

(iv) combining the DNA sequences encoding each CDR present in the naive library, optionally modified by mutation or replacement, at least in part, by synthetic sequences, with framework primers showing anchor region homology to form a range of CDR primers;

(v) assembling nucleic acid sequences to create a VHH repertoire by random recombination of the range of CDR primers with the amplified framework regions using a technique of splicing by overlap extension allowing fragment fusion at the annealing anchor regions.

In another aspect, the invention provides a method for the preparation of antibody fragments derived from a non-immunised source having specificity for a target antigen comprising screening an expression library as set forth above for antigen binding activity and recovering antibody fragments having the desired specificity.

30

The nucleic acid sequences encoding the heavy chain variable domains for use according to the invention may conveniently be cloned into an appropriate expression vector with allows fusion with a surface protein. Suitable vectors which may be used are well known in the art and include any DNA molecule, capable of

replication in a host organism, into which the nucleic acid sequence can be inserted. Examples include phage vectors, for example lambda, T4 or filamentous bacteriophage vectors such as M13. Alternatively, the cloning may be performed into plasmids, such as plasmids coding for bacterial membrane proteins or eukaryotic virus vectors.

The host cell may be prokaryotic or eukaryotic but is preferably bacterial, particularly *E. coli*.

10

The expression library according to the invention may be screened for antigen binding activity using conventional techniques well known in the art as described, for example, in Hoogenboom, Tibtech, 1997 (15), 62-70. By way of illustration, bacteriophage displaying a repertoire of nucleic acid sequences according to the invention on the surface of the phage may be screened against different antigens by a 'panning' process (see McCaterty, Nature, 348, (1990), 552-554) whereby the heavy chain variable domains are screened for binding to immobilised antigen. Binding phage are retained, eluted and amplified in bacteria. The panning cycle is repeated until enrichment of phage or antigen is observed and individual phage clones are then assayed for binding to the panning antigen and to uncoated polystyrene by phage ELISA.

25

As an indication of the binding affinities of antibodies that result from the screening described in the invention; dissociation constants for the VHHs recognising a protein antigen will typically be less than 100nM, preferably less than 75nM, more preferred less than 50nM, still more preferred at less than 40nM, most preferred less than 25nM.

The present invention therefore provides an expression library characterised in that superior binding affinity is achieved on screening than in many conventional dual chain antibody

expression libraries or single domain libraries based on variable domains derived from immunoglobulins which are not naturally devoid of light chains.

- 5 The invention further provides the use of a non-immunised source of synthetic and semi-synthetic nucleic acid sequences encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains to prepare an antibody, or fragment thereof, having binding specificity for a  
10 target antigen.

By means of the invention, antibodies, particularly fragments thereof, having a specificity for a target antigen may conveniently be prepared by a method which does not require the  
15 donor previously to have been immunised with the target antigen. The method of the invention provides an advantageous alternative to hybridoma technology, or cloning from B cells and spleen cells where for each antigen, a new library is required.

- 20 The present invention may be more fully understood with reference to the following description, when read together with the accompanying drawings.

Example 1. Construction of the naive VHH library.

25

1.1 Isolation of gene fragments encoding llama HC-V domains

A blood sample of about 200ml was taken from an non-immunised Llama and an enriched lymphocyte population was obtained via Ficoll (Pharmacia) discontinuous gradient centrifugation. From  
30 these cells, total RNA was isolated by acid guanidium thiocyanate extraction (e.g. via the method described by Chomczynski and Sacchi, Anal. Biochem, 162, 156-159 (1987)). After first strand cDNA synthesis (e.g. with the Amersham first strand cDNA kit), DNA fragments encoding VHH fragments and part

of the long or short hinge region where amplified by PCR using specific primers:

5 V<sub>H</sub> - 2B 5'-AGGTSMARCTG**CAG**SAGTCWGG-3'  
*Pst*I  
 (see SEQ. ID. NO: 1)

PCR.162:

10 5'-CATGCCATGACTCGCGGCCAGCCGGCCATGGCCSAGGTSMARCTG**CAG**SAGTCWGG-3'  
*Sfi*I  
 (see SEQ. ID. NO: 2)

S =C and G, M = A and C, R = A and G , W =A and T,

15 Lam-07:5'-AACAGTT**AAGCTT**CCGCTTGCGGCCGCGGAGCTGGGGTCTTCGCTGTGGTGCG-3'  
*Hind*III *Not*I  
 (see SEQ. ID. NO: 3)

20 Lam-08:5'-AACAGTT**AAGCTT**CCGCTTGCGGCCGCTGGTTGTGGTTTTGGTGTCTTGGGTT-3'  
*Hind*III *Not*I  
 (see SEQ. ID. NO: 4)

Upon digestion of the PCR fragments with *Pst*I (coinciding with codon 4 and 5 of the VHH domain, encoding the amino acids L-Q) and *Not*I (located at the 3'-end of the VHH gene fragments), the DNA fragments with a length between 300 and 400bp (encoding the VHH domain, but lacking the first three and the last three codons) were purified via gel electrophoresis and isolation from the agarose gel. *Not*I has a recognition-site of 8 nucleotides and it is therefore not likely that this recognition-site is present in many of the created PCR fragments. However, *Pst*I has a recognition-site of only 6 nucleotides. Theoretically this recognition-site could have been present in 10% of the created PCR fragments, and if this sequence is conserved in a certain class of antibody fragments, this group would not be represented in the library cloned as *Pst*I-*Not*I fragments. Therefore, a

second series of PCR was performed, in which the primary PCR product was used as a template (10ng/reaction). In this reaction the 5 prime VH2B primer was replaced by PCR162. This primer introduces a *Sfi*I recognition-site (8 nucleotides) at the 5 prime end of the amplified fragments for cloning. Thus, a total of 24 different PCR products were obtained, four (short and long hinge, *Pst* I/*Not* I and *Sfi* I/*Not* I) from each Llama. Upon digestion of the PCR fragments with *Sfi*I (upstream of the HC-V coding sequence, in the pelB leader sequence) and *Not*I, the DNA fragments with a length between 300 and 400bp (encoding the HC-V domain) were purified via gel electrophoresis and isolation from the agarose gel.

#### 1.2 Construction of HCV Library in pHEN.5

The *Pst* I/*Not* I or *Sfi* I/*Not* I - digested fragments were purified from agarose and inserted into the appropriately digested pHEN.5 vector. Prior to transformation, the ligation reactions were purified by extraction with equal volumes of phenol/chloroform, followed by extraction with chloroform only. The DNA was precipitated by addition of 0.1 volume 3M NaAc pH5.2 and 3 volumes ethanol. The DNA pellets were washed x2 with 1ml 70% ethanol, dried and resuspended in 10µl sterile milliQ water. Aliquots were transformed into electrocompetent *E. coli* XL1-Blue (Stratagene) by electroporation, using a Bio-Rad Gene Pulser. The protocol used was as recommended by Stratagene. The final library, consisting of approximately  $7.8 \times 10^6$  individual clones, was harvested by scraping the colonies into 2TY + Ampicillin (100ug/ml) + Glucose (2% w/v) culture medium (35-50ml each). Glycerol stocks (30% v/v) and DNA stocks were prepared from these and stored at -80°C.

## Example 2 Construction of the synthetic VHH library

Building on sequence data obtained from 200 individual clones randomly selected from the naive library 'anchor regions' in the framework sequences, immediately flanking the CDR regions were identified. These anchor regions were selected based on their high degree of conserved residues and the ability of primers based on these sequences to amplify most if not all of the approx.  $7.8 \times 10^6$  framework regions present in the naive library.

These anchor regions are used to amplify the framework regions individually yielding 5 different types/classes of fragments (F1/F2/F2c/F3/F4). The sequences of these primers are listed in Table 1.

### 2.1 PCR Considerations

All PCRs for amplification of the framework building blocks and assembly of the full length VHH genes were carried out using conditions and enzymes as described in Jespersen et al (1997). The conditions chosen utilised a mixture of Taq and Pfu (2 units and 1 unit, respectively in a 100 $\mu$ l reaction). The proof-reading activity of Pfu minimises the introduction of errors and, more importantly, removes the non-templated nucleotides added at the 3' terminus of PCR products amplified by Taq Polymerase. The presence of these nucleotides would result in the introduction of point mutations at the junctions of each building block in the full length VHH gene when assembled by overlap extension reactions.

### 2.2 Framework PCRs (F1, F2, F2c, F3 and F4)

Framework building blocks were amplified using naive library target DNA with the primers shown in Table 1. All framework and assembly DNA fragments were excised from agarose gels and purified by using the Qiaex extraction kit (Qiagen).

### 2.3 Assembly Reactions

CDR primers were designed on the basis of information derived from the analysis of naive library clone sequences, wherein  
5 largely conserved residues within the CDRs of such clones were assumed to perform a structural role and were maintained while sequence variability was designed in other areas.

The CDR Primers used in the construction of the VHH synthetic  
10 and semi-synthetic libraries are shown in Table 2 and a schematic representation of the assembly process is shown in Figure 1. Figure 2 gives an overview of the length and variability allowed in the CDR regions.

15 All primers used in CDR randomisation were synthesised leaving the trityl group on and full length primers were purified using cartridges and protocol supplied by Perkin Elmer. For every assembly step it was necessary to carry out 50-400 individual 100µl amplification reactions so that after Qiaex purification,  
20 enough material was available for the next step.

Calculations of the amount of 'virgin' i.e. primary assembled non-amplified sequences required to represent all potential variability (contained within the NNKs) for the early steps in  
25 the assembly of the VHH gene were made. It was ensured that more than this amount of material was used for further amplification at each assembly step (See 1.7).

Table 3 shows the entire matrix of fragments required for both  
30 libraries. For the synthetic library 70% of the library was made up of fragment H (which contains CD2-9) assembled onto all of the CD3 primer lengths, 20% fragment I (which contained CD2-10) on all of the CD3 lengths and 10% Fragment J on the longer CD3 lengths (14-24 amino acids). The length distributions were



modelled on the lengths found within the 200 randomly selected naive VHH sequences.

#### 2.4 Initial Assemblies (A, B, C, D, E and K5 to K24)

5 The primers encoding the randomised CDR regions were assembled with purified Framework DNA by overlap extension reactions, using large amounts of DNA and a small number of cycles to ensure maximum yields of 'virgin' i.e. non-amplified product. Assembly of CDR Primers onto purified framework DNA (Assemblies  
10 A to E and K5 to K24) was carried out for 10 cycles with 60ng of purified Framework DNA and 60 pmol of CDR Primer. Then a small aliquot was removed to estimate the amount of non-amplified material followed by a further 5 cycles with CDR primer (30 pmol; 1µl) and the relevant outside primer (45 pmol; 3µl) for  
15 each assembly (i.e. F4 for A; F4c for B; F6 for C, D and E and 170 for K5 to K24).

#### 2.5 Initial assemblies for the semi-synthetic library (N)

The fragment N (see Table 3) was amplified from target DNA (2µl)  
20 from each of 21 naive sub-libraries using primers F1 and F6 (45 pmol in a 100µl reaction).

#### 2.6 Intermediate Assemblies (F, G, H, I and J)

For later assemblies F (A onto F1), G (B onto F1), H (F onto C),  
25 I (F onto D) and J (G onto E) 10 cycles of amplification were carried out using the conditions described above and 200ng of relevant assembly and framework/assembly (see Table 3). Then a further 5 cycles were carried out with outside primers (3µl of 15 pmol/µl) (288 and F2 for F; 288 and F2c for G; 288 and F6 for  
30 H, I and J).

#### 2.7 Final Assemblies (P5-24, Q5-24, R5-24 and S5-24)

For each assembly: 100µl reactions were set up containing: 40-  
100ng of K5 to K24 product plus 100-200ng H, I, J, or N  
35 (ensuring excess of the larger fragment in molar terms). One to

four of these reactions was carried out per required assembly, depending on amount of final product needed. The total amount of 'virgin' material in 100-400 $\mu$ l total reactions was estimated and then 3 $\mu$ l of each outside primer (170, 288; 15 $\mu$ mol/ $\mu$ l) was added to each 100 $\mu$ l reaction and then amplification was continued for a further 10 cycles. Yields of 'virgin' full length assembled material (before any amplification with outside primers) for all 71 fragments ranged from 40ng to 400ng, depending upon amounts and efficiency. This was more than sufficient to represent all potential variants as single transformants as 300ng of a 400-500 base fragment is more than  $10^{11}$  individual molecules (see Example 2.1 for the total size of the libraries).

#### 2.8 Scale-up amplification of full length VHH for cloning

The final number of PCRs required to give the desired mixture amount for cloning of any particular fragment was calculated based on the natural distribution of CDR length and composition (Table 4). The actual mixture distribution was influenced to a certain extent by different yields obtained for different fragments.

Large-scale amplification reactions were carried out by adding 5 $\mu$ l-20 $\mu$ l of the first PCR reactions, (after 10 cycles overlap extension and 10 cycles outside primers) to 170/288 PCRs (- 25 cycles with 2 mins elongation at 72 degrees). Total final amplifications of the 71 fragments (51 for the Synthetic library and 20 for the Semi-Synthetic library) were pooled, precipitated and run on 1% agarose gels; the full-length VHH gene product was excised and purified by Qiaex extraction. Yields after purification were approx. 1  $\mu$ g per 100 $\mu$ l PCR Reaction.

#### 2.9 Construction of the VHH Libraries in pHEN.5

The 71 gel-purified DNA fragments encoding the VHH genes for the libraries were digested with SfiI (upstream of the VHH coding

sequence, in the *pelB* leader sequence) and *NotI* (located at the 3'-end of the VHH gene fragments). Transformation of 100µl of electrocompetent XL1-Blue (Stratagene) *E. coli* with 5µg of digested, purified pHEN.5 vector and 0.5µg of purified, digested test fragment (close to saturation) gave approx.  $8 \times 10^7$  transformants. 1000 x 100µl electroporations were split between the two libraries.

The amount of purified, digested fragment was estimated for each before insertion into *SfiI/NotI* digested pHEN.5 and the number of ligation reactions for each fragment was calculated depending upon the amount of components in the mix. Prior to transformation, the ligation reactions were purified by extraction with equal volumes of phenol/chloroform, followed by extraction with chloroform only. The DNA was precipitated by addition of 0.1 volume 3M NaAc pH 5.2 and 3 volumes ethanol. The DNA pellets were washed x2 with 1ml 70% ethanol, dried and resuspended in 10µl sterile milliQ water. Aliquots were transformed into electrocompetent *E. coli* XL1-Blue (Stratagene) by electroporation, using a Bio-Rad Gene Pulser. The protocol used was as recommended by Stratagene.

#### 2.10 Large-scale phage rescue of synthetic VHH Libraries

The final libraries consisted of  $6 \times 10^{10}$  individual clones (Synthetic library) and  $4.4 \times 10^{10}$  clones (Semi-Synthetic library). The numbers of transformants for each individual fragment were calculated by pooling and titering of recovered transformants. Transformed XL1-Blue were then harvested in solution by increasing the volume of each fragment pool by 10-fold with 2TY containing Ampicillin (100µg/ml) and Glucose (2% w/v) followed by 3 hours of growth at 37°C with shaking. Half of this total culture volume was pooled, grown for a further 2 hours at 37°C with shaking, spun and stored as concentrated glycerol stocks.

The remaining half of the library was diluted 2-fold with 2TY/Ampicillin/Glucose and helper phage were added.

5 Super-infection was allowed to occur for 30 mins at 37°C without shaking, followed by incubation with shaking for 30 mins. The 10 litres were spun at 5000 rpm for 20 mins and the pellets were pooled. Each pellet was resuspended in 20mls of 2TY containing Ampicillin (100ug/ml) and Kanamycin (50ug/ml) and pooled into one 2L flask.

10

The final volume was made up to 20L with 2TY/Amp/Kan and the cultures were grown overnight, shaking at 37°C. Phage were harvested by two consecutive precipitations with 1/5 volume 20% Polyethylene Glycol 8000, 2.5 M NaCl and several aliquots were used directly for panning (see below). The remaining phage aliquots (180x1ml for each library) were resuspended in PBS/30% glycerol and stored at -80°C.

### 20 Example 3 Selection of VHH fragments with binding affinity

#### 3.1 Panning of the library using solid phase immobilised antigens

##### Antigens:

25 Five 'antigens' were used to screen the synthetic library. These were Lactate Oxidase (LOX), Starch Branching Enzyme II (SBE II), Classical VHH antibody, a mix of Polyphenols, and Haem conjugated to Bovine Serum Albumin. Panning of phages displaying VHHs was carried out as described below.

30

##### Panning and Phage Rescue

Aliquots of phage (1ml;  $\sim 10^{13}$  phage particles resuspended in 2% Marvel [plus 2% OVA or BSA]) from the large scale phage rescue (see above) were incubated overnight with the relevant sensitised panning tubes (Nunc Maxisorb Immunosorb). Unbound

35

phage were removed by washing the tube 20 times with PBS-T followed by 20 washes with PBS. The bound phages were eluted by adding 1mL elution buffer (0.1M HCL/glycine pH 2.2/1mg/mL BSA). The elution mixture was neutralised with 60µL 2M Tris, and the  
5 eluted phages were added to 9mL log-phase *E. coli* XL-1 Blue. Also 4mL log-phase *E. coli* XL-1 Blue were added to the immunotube. After incubation at 37°C for 30 minutes to allow infection, the 10mL and 4mL infected XL-1 Blue bacteria were pooled and plated onto SOBAG plates. Following growth overnight  
10 at 37°C the clones obtained from the antigen sensitised tubes were harvested and used as starting material for the next round of panning, or alternatively individual colonies were assayed for specific antigen binding activity.

15 To continue panning, an aliquot (150µl) of the overnight culture was added to 15ml of 2TY containing Ampicillin (100µg/ml) and Glucose (2% w/v) and allowed to grow until log-phase ( $A_{600} = 0.3-0.5$ ), at which point  $4.5 \times 10^9$  pfu M13K07 helper phage were added. After infection for 30 minutes at 37°C (without shaking)  
20 the infected cells were spun down (5000 rpm for 10 minutes) and the pellet was resuspended in 200mL 2xTY/Amp/Kan. After incubation with shaking at 37°C overnight, the culture was spun and the phages present in the supernatant were precipitated by adding 1/5 volume PEG/NaCl (20% Polyethylene glycol 8000, 2.5M  
25 NaCl). After incubation on ice-water for 1 hour the phage particles were pelleted by centrifugation at 8000 rpm for 30 minutes. The phage pellet was resuspended in 20mL water and re-precipitated by adding 4mL PEG/NaCl solution. After incubation in ice-water for 15 minutes the phage particles were pelleted by  
30 centrifugation at 5000 rpm for 15 minutes and resuspended in 2mL PBST with 2% Marvel (plus 2% OVA or BSA). Panning results are outlined in Table 5.

Example 4 Identification of individual HC-V fragments with binding activity

Individual bacterial colonies were picked (48 from pans 2 and 3, for all antigens) using sterile toothpicks and added to the wells of 96-well microtitre plates (Sterilin) each containing 100µl of 2TY, 1% (w/v) glucose and ampicillin (100mg/ml). After allowing the cultures to grow o/n at 37°C, 20µl aliquots from each well of these 'masterplates' were added to the wells of fresh microtitre plates each containing 200µl of 2TY, 1% glucose, 100mg/ml ampicillin, 10<sup>9</sup> M13KO7 helper phage. Infection at 37°C for 2.5h was followed by pelleting the cells and resuspending the infected cells in 200µl of 2TY containing ampicillin (100mg/ml) and kanamycin (25mg/ml). Following o/n incubation at 37°C, the phage-containing supernatants (100µl) were added to the wells of Sterilin microtitre plates containing 100µl/well of the appropriate blocking buffer (same buffer used as during panning reactions). Pre-blocking of the phage was carried out in these plates for 30 mins at room temp. After 30 minutes at RT, 100µl of phage supernatant was added to the wells of a Greiner High Bind ELISA plate coated with the corresponding antigen, and to the wells of an uncoated plate. After 2h incubation at 37°C unbound phages were removed, and bound phages were detected with rabbit anti-M13 (in house reagent) followed by incubation with a goat anti-rabbit alkaline phosphatase conjugate. The assays were developed with 100µl/well of p-nitrophenyl phosphate (1mg/ml) in 1M diethanolamine, 1mM MgCl<sub>2</sub>, pH 9.6 and the plates were read after 5-10 mins at 410nm. Results are outlined in Table 6.

30

Example 5 Identification of individual VHH binders using solution phase panning

An alternative method has been used for the screening of the semi-synthetic library using the enzyme amylase as a target.

The antigen was biotinylated and panned using streptavidin-coated magnetic beads. High affinity binders were then selected by dropping the antigen concentration from 100nM to 30nM. A panel of highly specific antibody fragments were isolated and the Kd values determined using Pharmacia BiaCore SPR technology. The VHHs were captured on an NTA sensor chip via the Histidine-tag present at the C-terminus. Various concentrations (5 to 200nM) of amylase were then passed over the sensor chip and the equilibrium constants were measured using the standard evaluation software. Results are outlined in Table 7.

Table 1. Framework primers

Code	Description	Sequence
F1	PelB leader	ATT GCC TAC GGC AGC CGC TG
F2	3' FR-1	TCC AGA GGC TGC ACA GGA GA
F3	5' FR-2	TGG T(A/T)C CGC CAG GCT CCA GG
F4	3' FR-2	GC GAC (C/A)AA CTC GCG CT(G/C) CTT
F3c	5' FR-2c	TGG TTC CGC CAG GCC CCA GG
F4c	3' FR-2c	ACA TGA GAC C(C/G)C CTC (G/A)CG CTC
F5	5' FR-3	TAT GCA GAC TCC GTG AAG GGC CG
F6	3' FR-3	ACA GTA ATA (G/A)AC GGC CGT GTC
F7	5' FR-4	TGG GGC CAG GGG ACC C(A/T)G GTC
F8	myc-tag	TTC AGA TCC TCT TCT GAG ATG AG

Table 2. CDR primers

Code	Description	Sequence
CD-1	CDR-1 (5'-3')	T(T/C) TCC TGT GCA GCC TCT GGA AG(T/C/A) A(C/T) (C/T) TT(T/C/G) AG(T/C/A) NNK NNK NNK ATG GGT TGG T(A/T)C CGC CAG GCT CCA GG
CD-1a	CDR-1 (5'-3')	T(T/C) TCC TGT GCA GCC TCT GGA G(T/A)TC A(C/T) (C/T) TT(T/C/G) GAT NNK TAT NNK ATT GGT TGG TTC CGC CAG GCC CCA GG
CD-2-9	CDR-2 9 a.a.	AAG (C/G)AG CGC GAG TT(T/G) GTC GCA NNK ATT (T/A)CT NNK GGT GGT NNK ACA NNK TAT GCA GAC TCC GTG AAG GGC CG
CD-2-10	CDR-2 10 a.a.	AAG (C/G)AG CGC GAG TT(T/G) GTC GCA NNK ATT (T/A)CT NNK NNK GGT GGT NNK ACA NNK TAT GCA GAC TCC GTG AAG GGC CG
CD-2-10c	CDR-2c	GAG CG(C/T) GAG G(G/C)G GTC TCA TGT TGT ATT (T/A)CT NNK NNK GAT GGT NNK ACA NNK TAT GCA GAC TCC GTG AAG GGC CG
CD-3-2	CDR-3 5 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G(A) (C/A)T GCC NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-3	CDR-3 6 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G(A) (C/A)T GCC NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-4	CDR-3 7 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G(A) (C/A)T GCC NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-5	CDR-3 8 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G(A) (C/A)T GCC NNK NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-6	CDR-3 9 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G(A) (C/A)T GCC NNK NNK NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-7	CDR-3 10 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G(A) (C/A)T GCC NNK NNK NNK NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-8	CDR-3 11 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G(A) (C/A)T GCC NNK NNK NNK NNK NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-9	CDR-3 12 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G(A) (C/A)T GCC NNK NNK NNK NNK NNK NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-10	CDR-3 13 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G(A) (C/A)T GCC NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-11	CDR-3 14 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G(A) (C/A)T GCC NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC



Code	Description	Sequence
CD-3-12	CDR-3 15 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G/A) (C/A) T GCC NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-13	CDR-3 16 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G/A) (C/A) T GCC NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-14	CDR-3 17 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G/A) (C/A) T GCC NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-15	CDR-3 18 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G/A) (C/A) T GCC NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-16	CDR-3 19 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G/A) (C/A) T GCC NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-17	CDR-3 20 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G/A) (C/A) T GCC NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-18	CDR-3 21 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G/A) (C/A) T GCC NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-19	CDR-3 22 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G/A) (C/A) T GCC NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-20	CDR-3 23 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G/A) (C/A) T GCC NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC
CD-3-21	CDR-3 24 a.a.	GAC ACG GCC GT(C/T) TAT TAC TGT G/A) (C/A) T GCC NNK TAC TGG GGC CAG GGG ACC CA(G/T) GTC

N=G/A/T/C

K=G/T

Table 3. Steps in the Assembly of the Synthetic VHH Library  
(also see Figures 1 and 2)

Assembly Name	Step Required
<b>Synthetic Library</b>	
A	CD1 onto F2
B	CD1c onto F2c
C	CD2-9 onto F3
D	CD2-10 onto F3
E	CD2-10c onto F3
F	CD1-F2 (A) onto F1
G	CD1c-F2c (B) onto F1
H	F1-CD1-F2 (F) onto CD2-9-F3 (C)
I	F1-CD1-F2 (F) onto CD2-10-F3 (D)
J	F1-CD1c-F2c (G) onto CD2-10c-F3 (E)
K5 to K24	CD3-5, CD3-6, CD3-7 up to CD3-24 onto F4
P5 to P24	H onto K5 to K24
Q5 to Q24	I onto K5 to K24
R5 to R24	J onto K5 to K24
<b>Semi-Synthetic Library</b>	
N	F1-CD1-F2-CD2-F3 amplified from naive library DNA using F1 and F6
S5 to S24	N onto K5 to K24

Table 4. Number of Scale-up PCRs for Final Assemblies


		CD 3-5	CD 3-6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
Synthetic																						
	70%	H	5	5	10	30	50	55	60	70	80	85	90	85	70	55	40	20	10	10	5	5
	20%	I	5	5	5	10	10	15	20	20	25	30	25	20	15	10	10	5	5	5	5	5
	10%	J											10	10	10	10	10	10	10	10	10	10
Semi-synthetic	N	10	10	20	50	60	70	80	90	100	120	130	110	90	80	60	40	20	20	10	10	

Table 5. Synthetic(S) and Semi-Synthetic (SS) Library  
Panning Results

Panning Antigen	Pan 1	Pan 2	Pan 3	Pan 4	Pan 5
LOX (SS)	ND	2- fold	12- fold	-	-
SBE II (S)	ND	NONE	NONE	8-fold	8-fold
HCV-Classical (SS)	ND	1200-fold	10000-fold	-	-
Polyphenols (SS)	ND	52-fold	32-fold	-	-
Haem (SS)	ND	500-fold	1000-fold	-	-

5

Table 6. Percentage of VHH phage clones which specifically  
recognise immobilised antigen

Panning Antigens	Pan 2	Pan 3	Pan 4	Pan 5
LOX (SS)	2%	38%	-	-
SBE II (S)	0%	4%	48%	60%
HCV-Classical (SS)	31%	54%	-	-
Polyphenols (SS)	23%	9%	-	-
Haem (SS)	77%	79%	-	-

10

Table 7. Equilibrium Constants for VHHs recognising Amylase

VHH Number	Equilibrium Constants (nM)
11G	6
12A	11
3A	16
7E	22
3F	34
5F	39
9C	41

**CLAIMS**

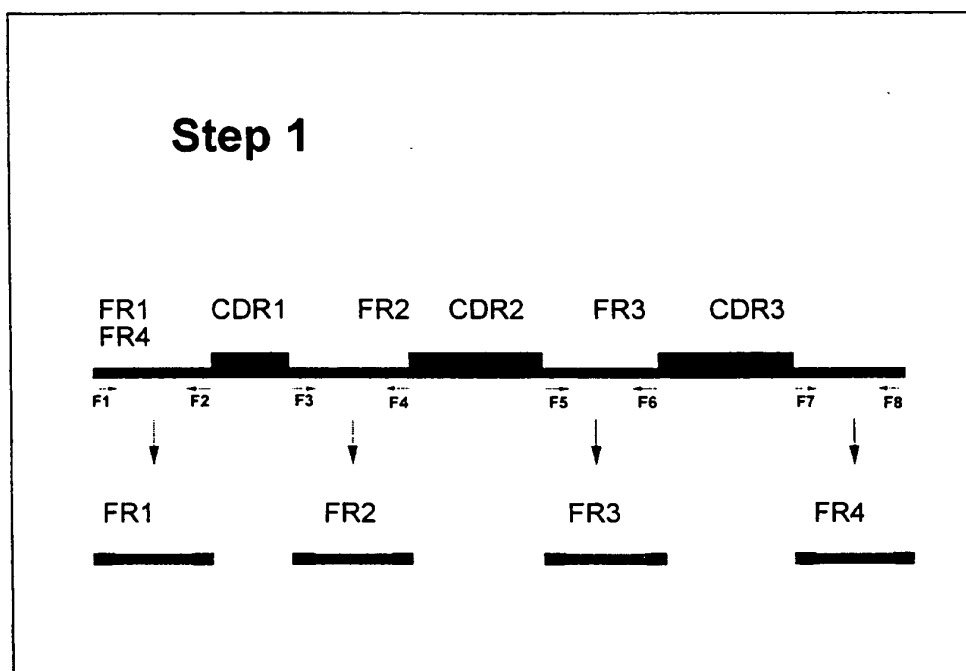
1. An expression library comprising a repertoire of nucleic acid sequences, which sequences are not cloned from an immunised source, each nucleic acid sequence encoding at least part of a variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains (VHH) wherein the extent of sequence variability in said library is enhanced compared to the corresponding naïve expression library repertoire by the introduction of mutations in one or more of the complementarity determining regions (CDRs) of said nucleic acid sequences or by generating alternative combinations of CDR and framework sequences not naturally present in the naïve library repertoire.
2. A library according to claim 1, wherein the nucleic acid sequences are modified by random or partially random mutation of one or more of the CDR sequences.
3. A library according to claim 1 or claim 2, wherein at least part of one or more of the CDR sequences is replaced by synthetic nucleic acid sequences.
4. A library according to any one of claims 1 to 3, wherein at least part of each of the CDR sequences is replaced by synthetic nucleic acid sequences.
5. A library according to claim 1, wherein VHH sequences comprising alternative CDR and framework sequence combinations are generated by random recombination of fragments of VHH sequences derived from a naïve library or a library according to any one of claims 2 to 4.

6. A library according to any one of claims 1 to 5, wherein conserved residues within the CDR sequences are retained in the modified nucleic acid sequences.
- 5 7. A library according to any one of claims 1 to 6 wherein sequence variability present in the naïve library repertoire in those parts of the VHH domain other than the CDRs is conserved.
- 10 8. An expression library according to any one of claims 1 to 7, wherein the variable domain of a heavy chain derived from an immunoglobulin naturally devoid of light chains is derived from a camelid immunoglobulin.
- 15 9. Use of a naïve VHH expression library to prepare an expression library according to any one of claims 1 to 8.
10. A method for preparing a library according to any one of claims 1 to 8 comprising the steps:
  - 20 (i) taking sequence data obtained from a number of cDNA clones randomly selected from a naïve VHH library;
  - (ii) identifying a series anchor regions which show  
25 substantial conserved homology within said sequence data and on which basis framework primers with a capacity to amplify framework regions of the naïve library target DNA can be constructed;
  - 30 (iii) amplification from a non-immunised source of a maximal number of different framework regions using primers from step (ii);
  - 35 (iv) combining the DNA sequences encoding each CDR present in the naïve library, optionally modified by mutation

or replacement, at least in part, by synthetic sequences, with framework primers to form a range of CDR primers;

- 5           (v) assembling nucleic acid sequences to create a VHH repertoire by random recombination of the range of CDR primers with the amplified framework regions using a technique of splicing by overlap extension.
- 10   11. Method for the preparation of antibody fragments derived from a non-immunised source having specificity for a target antigen comprising screening an expression library according to any of claims 1 to 9 for antigen binding activity and recovering antibody fragments having the
- 15           desired specificity.

Figure 1.





## Step 2A

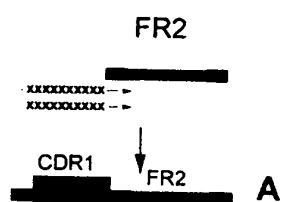


Fig.1B.

Step2B

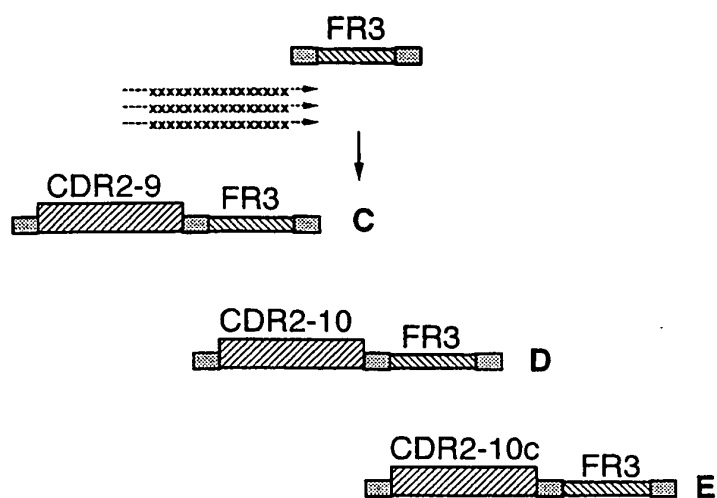


Fig.1C.

Step2C

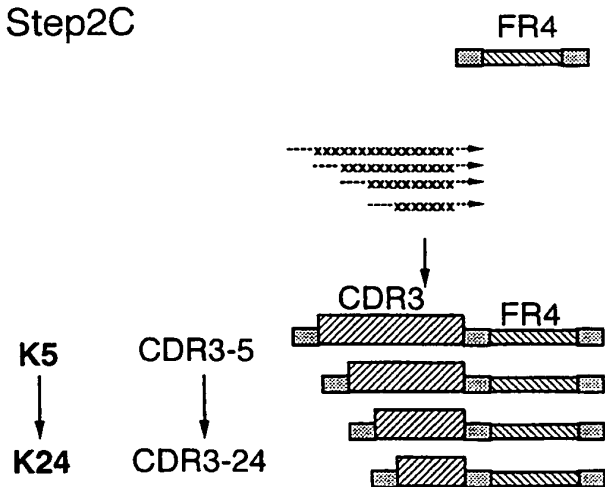


Fig.1D.

Step 3

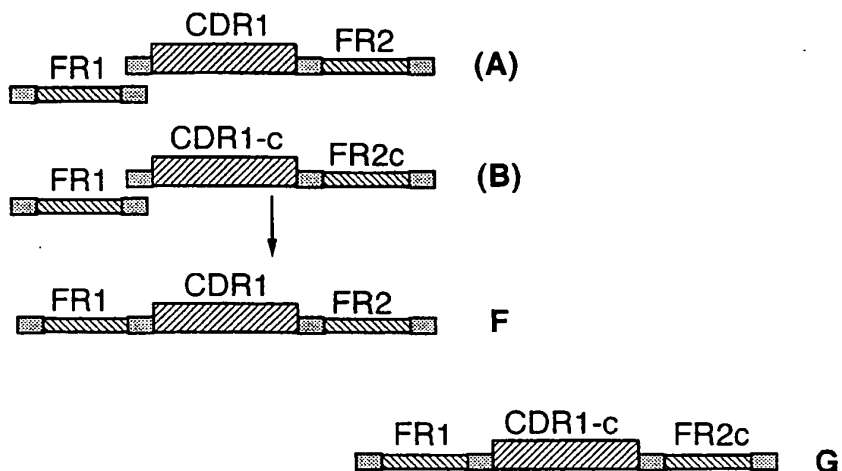


Fig.1E.

Step 4

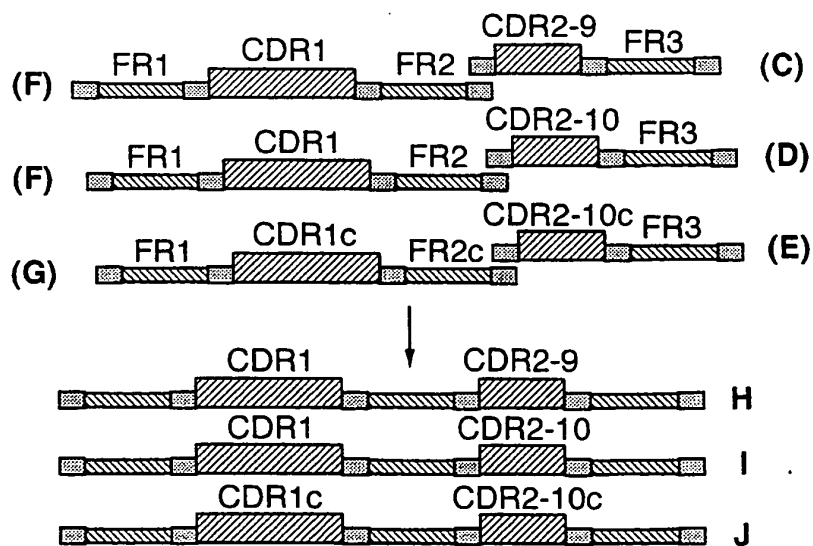


Fig.1F.

Step 5A

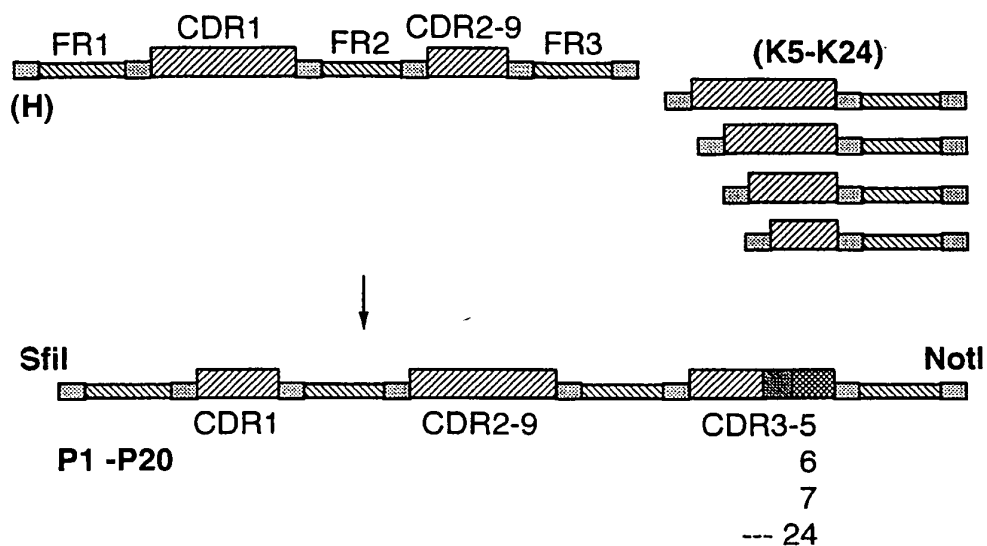


Fig.1G.

Step 5B

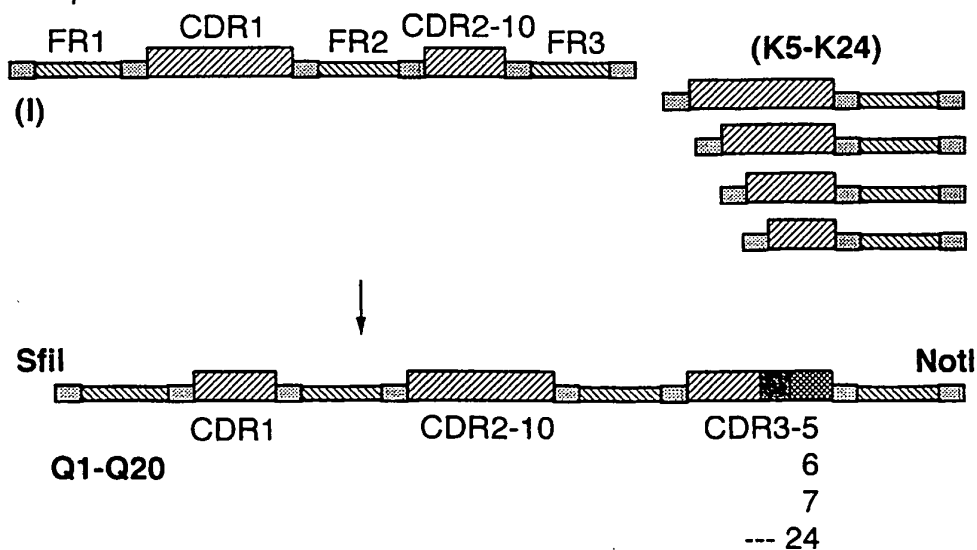


Fig.1H.

Step 5C

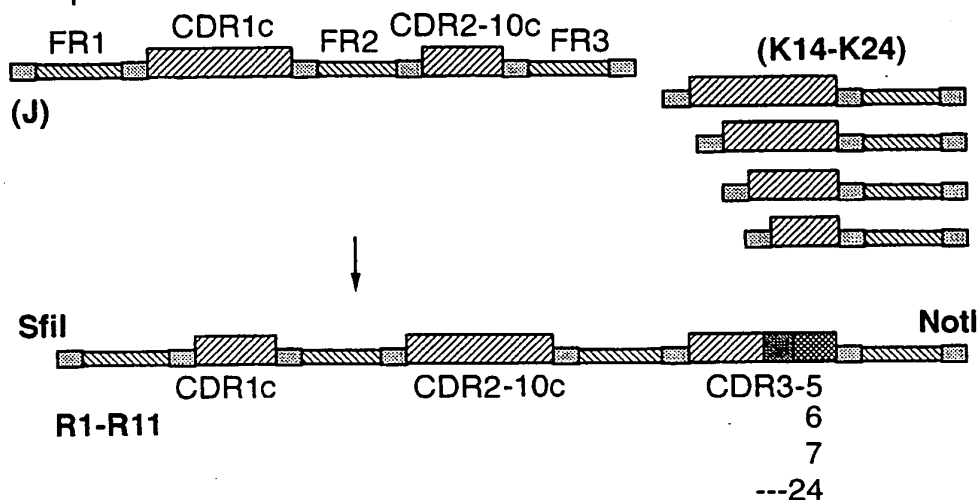


Fig.1I.

Step 5D

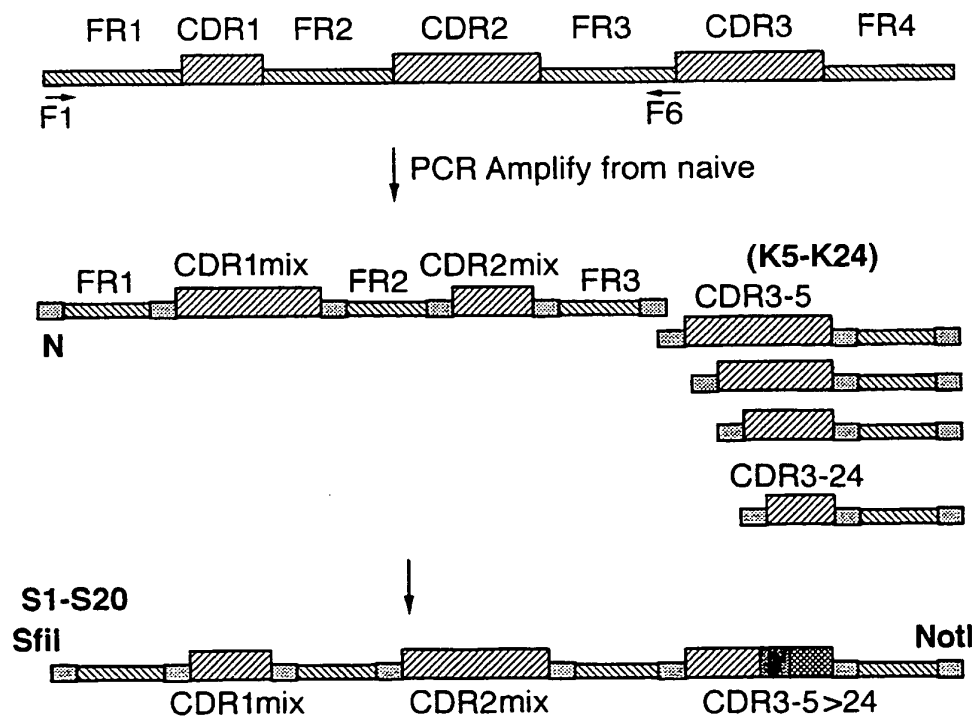
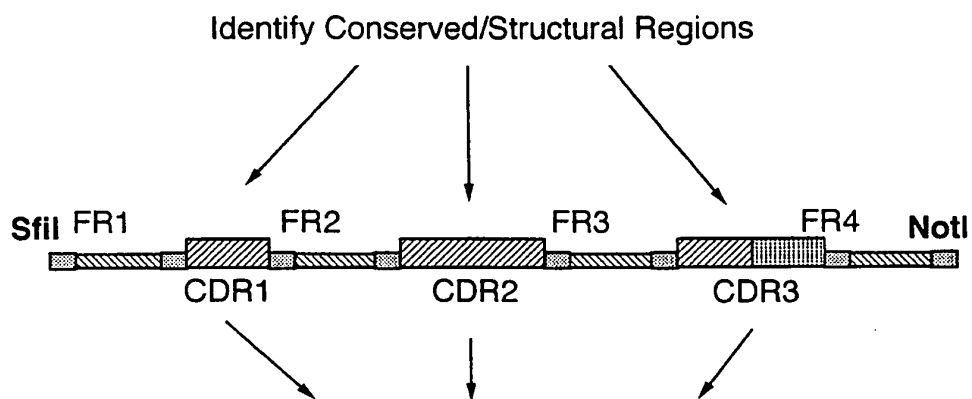


Fig.2.



Size	9	9 or 10	5-24
Random	3	4 or 5	2-19
Partly Random	4	1	1